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EVIDENCE FOR PHASE SEPARATION IN THE MEMBRANE OF AN OSMOTICALLY STABILIZED FATTY ACID AUXOTROPH OF *E. COLI* AND ITS BIOLOGICAL SIGNIFICANCE

HIDEO AKUTSU [a], YUZURU AKAMATSU [b], TOSHIO SHINBO [a,*], KAKU UEHARA [a,**], KATSUTADA TAKAHASHI [c] and YOSHIMASA KYOGOKU [a]

[a] *Institute for Protein Research, Osaka University, Suita, Osaka 565,* [b] *Department of Chemistry, National Institute of Health, Shinagawa, Tokyo 141 and* [c] *Laboratory of Biophysical Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591 (Japan)*

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Summary

1. An unsaturated fatty acid auxotroph of *Escherichia coli* accumulated a high content of saturated fatty acids in its membrane when it was cultured under osmotically stabilized conditions. The physicochemical properties of the phospholipid extracts and of the membrane fraction from the cells were investigated by means of proton magnetic resonance, infrared spectroscopy and differential scanning calorimetry.

2. Physicochemical studies indicate that the phospholipid bilayers in the membranes exhibit at least two phase transitions, a minor one at approx. 19°C and a major one at approx. 43°C. Between the two temperatures, gel and liquid crystalline domains co-exist. Moreover, even in the gel state, phospholipids seem to segregated into domains containing different proportions of unsaturated fatty acids.

3. The Arrhenius plot of β-galactoside transport rates is biphasic. The inflection point is at 22°C. This means that the appearance of the fluid region in the bilayer at approx. 19°C is important in the activation of membrane transport.

Introduction

Unsaturated fatty acid auxotrophs of *Escherichia coli* are suitable organisms for monitoring the influence of the physical properties of lipids on the func-

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[*] Present address: National Chemical Laboratory for Industry, Nishiyawata, Hiratsuka, Kanagawa 254, Japan.

[**] Present address: Department of Applied Chemistry, College of Engineering, University of Osaka Prefecture, Sakai, Osaka 591, Japan.

tions of cellular membranes. Their properties have been investigated by a variety of methods. In those studies, the correlations between the phase transition of the phospholipid bilayer and some functions of the biomembranes have been clarified by controlling the phase transition temperature with several kinds of supplied unsaturated fatty acids [1—3]. Furthermore, the segregation of membrane lipids and proteins was also shown in the auxotroph supplemented with linolenic acid [4]. However, the detailed properties of *E. coli* membranes with a fairly high content of saturated fatty acids have not yet been clarified, since *E. coli* with such membranes cannot grow under the normal culture conditions. As we reported earlier [5,6], *E. coli* K-12 UFA^{ts} can grow in the presence of 2% KCl at 42°C without oleic acid supplement. The content of saturated fatty acids in its membrane is more than 90% and is the largest observed in *E. coli* membranes as far as we know. The bilayer prepared from the extracted phospholipids shows a fairly sharp phase transition near the growth temperature. In the present work the physicochemical properties of the phospholipid bilayers and the membrane fraction were investigated in detail by means of proton magnetic resonance (^{1}H-NMR), infrared spectroscopy and differential scanning calorimetry (DSC). Furthermore, the biological significance of the observed multiple phase transitions is discussed in relation to the temperature dependence of the rate of β-galactoside transport across the membrane.

Materials and Methods

Extraction of phospholipids. E. coli K-12 UFA^{ts}, a temperature-sensitive unsaturated fatty acid auxotroph, was cultured overnight at 42°C in a 200-L fermenter in aerated synthetic minimum media [7] with 1% casamino acids (Difco, vitamin-free) and 0.25% glycerol, and further with 2% KCl as an osmotic stabilizer. The cells used in this work were from the same large culture in the previous study [6]. Whole phospholipids were extracted from the cells according to the method of Bligh and Dyer [8] and were purified by passing through a silicic acid column. The phospholipid fraction from the *E. coli* cells is called PL42 (KCl) hereafter.

Analysis of chemical composition. The molar ratios of the separated phospholipids were determined from phosphorus assay of the spots on the thin-layer chromatographic plots according to the method of Bartlett [9]. The fatty acid composition of methyl esters obtained by transesterification with HCl/methanol was determined according to the method described previously [6].

Physicochemical measurements. Sonicated vesicles were prepared as follows. The interior of a 10 ml test tube with a cone-shaped bottom was coated with a thin film of phospholipids by evaporating to dryness under vacuum. They were then taken up in ^2H_2O or H_2O. The mixture was cooled at 5°C for 20 min in a nitrogen atmosphere and sonicated for five 1 min bursts separated by 1 min intervals. A Branson sonifier, model B-12 (20 kHz), was used. p^{2}H and pH values were measured by a Hitachi-Horiba pH meter, model M-5, equipped with a microelectrode (Ingold Electrodes Inc.). For DSC, 11 mg of the phospholipid fraction in a small volume of chloroform was put in an aluminum DSC cell and evaporated to dryness under vacuum for 1 day. After the addition of 11 mg of

$H_2\bar{O}$, the cell was hermetically sealed and kept at 80° C for 1 h. The bulk *E. coli* membrane fraction used for the calorimetric experiments was obtained according to the method of Miura and Mizushima [10]. Subfractionation was not carried out. Intact cells and non-burst spheroplasts were removed by centrifugation at 2500 rev./min for 15 min. The measurements were performed on the pellet which was prepared by centrifugation at 150 000 × *g* after washing 3 times at 25 000 × *g* with 5 mM $MgCl_2$ solution.

^{1}H-NMR spectra of the phospholipid dispersions were recorded on a JEOL PFT-100 pulse Fourier transform NMR system locked on deuterium and equipped with temperature control apparatus. Temperature was determined by the calibrated chemical shifts of methanol and 1,3-propanediol. Infrared spectra were obtained with a Hitachi EPI-G3 infrared spectrophotometer. Solutions were put in shield cells of 0.05 mm thickness with CaF_2 windows. Temperatures were calibrated by the use of a thermocouple. Calorimetric experiments were performed in a Seiko heat leakage scanning calorimeter SSC-544 and a Rigaku Denki thermoflex differential scanning calorimeter. ^2H_2O (99.8%) was purchased from CEA (Commissariat a l'Energie Atomique).

Measurement of β-galactoside transport rates. An overnight culture at 28° C of *E. coli* K-12 UFA^{ts} was diluted to $7 \cdot 10^7$ cells/ml with medium (containing 2% KCl). The culture was incubated at 42° C for 5 h (approx. four generations) with 1 μM isopropyl-1-thio-β-galactoside to induce β-galactoside transport. β-Galactoside transport rates were measured by the release of *o*-nitrophenol from *o*-nitrophenyl galactoside according to the method of Wilson and Fox [11]. The cells were used without prior washing.

Results

Phase transitions of phospholipid bilayers

Proton magnetic resonance. The linewidths of the methylene signal of PL42 (KCl) at p^{2}H 8.8 were plotted as a function of $1/T$ (T, absolute temperature) in Fig. 1. Linewidths were determined as described in our previous paper [6]. On heating, fairly large changes of the linewidth and the peak height were observed at approx. 42° C. They were attributed to the main phase transition of the lipid bilayers of the PL42 (KCl) vesicles [6]. In addition, another change in the slope of the plotted curve can be seen at approx. 13° C. Similar features have been observed in the plots for several p^{2}H values between 4 and 10. The plots of the linewidths on cooling after incubation at 70° C for a few hours are also shown in Fig. 1. A change in the slope of the curve is seen at approx. 15° C. When the sample was kept for a few hours at 70° C, most of the PL42 (KCl) vesicles aggregated and gathered on the top of the sample solution. Thus the signals on cooling arose only from the transparent part of the solution. The aggregated phospholipids have so broad a linewidth that they did not contribute to the observed linewidths. The narrower linewidth of the cooling profile, compared with that of the heating profile, may be due to the decrease in the vesicle size. The results indicate that there are at least two kinds of vesicle, in one of which the transition temperature is at approx. 13° C and in the other at 42° C. As shown in the electron micrograph of the PL42 (KCl) vesicles given in the previous paper [6], there is distribution of vesicle sizes. The mean diameter

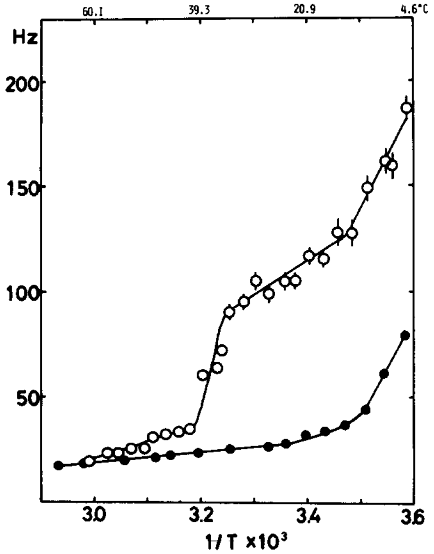

Fig. 1. Plot of the linewidth of the methylene proton signal (100 MHz) of PL42 (KCl) vesicles against the reciprocal of absolute temperatures, ○, heating; ●, cooling after incubation for several hours at 70°C.

of the larger vesicles is 1000 Å and that of the smaller ones is 300 Å. There is no appreciable change in the vesicle sizes between 5°C and 20°C, but sizes begin to change above 40°C.

A fatty acid analysis was carried out on the vesicles aggregated after incubation at 70°C and on those remaining in suspension. As shown in Table I, the amount of unsaturated fatty acids in the aggregate is negligible. This is strong evidence that one type of vesicle carries only traces of unsaturated fatty acids while the other type has many unsaturated fatty acids. The phase transition temperatures of the two vesicle types can be assigned as 42°C and 13°C,

TABLE I

FATTY ACID COMPOSITION OF AGGREGATE AND SUSPENSION

The phospholipid composition of PL42 (KCl) was: phosphatidylethanolamine 79.8%; cardiolipin 15.7% and phosphatidylglycerol 4.5%.

Fatty acid	Aggregate (mol%)	Suspension (mol%)
12 : 0	2.59	2.64
14 : 0	40.33	39.42
14 : 1	0.58	2.27
16 : 0	50.50	48.30
16 : 1	0	1.18
17Δ	3.14	3.03
18 : 1	0	0.29
19Δ	2.85	2.88
Total of unsaturated fatty acids	0.58	3.74
(+ fatty acids with cyclopropane)	6.57	9.65

respectively. It follows that the bilayer forms immiscible domains with different unsaturated fatty acid composition in the gel state at 5° C, the temperature at which sonications were performed. The values of the suspension in Table I do not truly represent the fatty acid composition of the suspension because it still contains many small aggregates which were impossible to remove further. It should be noted that the fatty acids with cyclopropane groups behave in a manner different from the unsaturated fatty acids here.

Infrared spectra. In order to see if the real origin of the major transition is the aggregation process, infrared spectroscopic studies have been done, since infrared spectra are insensitive to macroscopic changes such as aggregation. Infrared spectra of the sonicated vesicles of PL42 (KCl) in H_2O (20% w/v) were observed at different temperatures. The intensities of the band at 1470 cm^{-1} relative to those at 1380 cm^{-1} were plotted as a function of temperature. The 1380 cm^{-1} band is due to the methyl symmetric deformation vibration and was used as an internal reference since its intensity is expected to be independent of the conformation of hydrocarbon chains. The result is shown in Fig. 2. On heating, the intensity of the band at 1470 cm^{-1} decreases significantly in the region between 30 and 50° C and the midpoint of the change is at approx. 42° C. It is known that an infrared active band due to the methylene deformation vibration of a polyethylene chain is found at approx. 1470 cm^{-1} when the two neighboring methylene groups take the *trans* conformation. The band moves to a frequency lower than 1450 cm^{-1} in the *gauche* conformation [12—14]. Therefore, it can be concluded that the large change in the linewidth of the methylene signal in the ^{1}H-NMR spectrum is not due to the formation of lipid aggregates but to the phase transition of the hydrocarbon chains of phospholipids. Absence of the transition at a lower temperature in the infrared study is simply due to the low sensitivity of the method.

Differential scanning calorimetry. A multilamellar system is a better model of a biomembrane than a sonicated vesicle, because of the structural distortion due to the curvature of the vesicle. DSC on the PL42 (KCl) multilamellar system (50% H_2O) was performed. Its heating and cooling thermograms are shown in Fig. 3, where the temperature scanning rate is 10 K · min^{-1}. These thermograms showed good reproducibility in repeated measurements. The thermograms observed on cooling are sharper than those observed on heating. There is

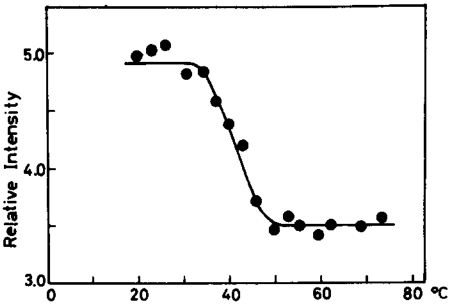

Fig. 2. Temperature dependence of the relative intensity of the methylene scissoring band to the methyl deformation band (I_{1470}/I_{1380}) of PL42 (KCl) vesicles in H_2O.

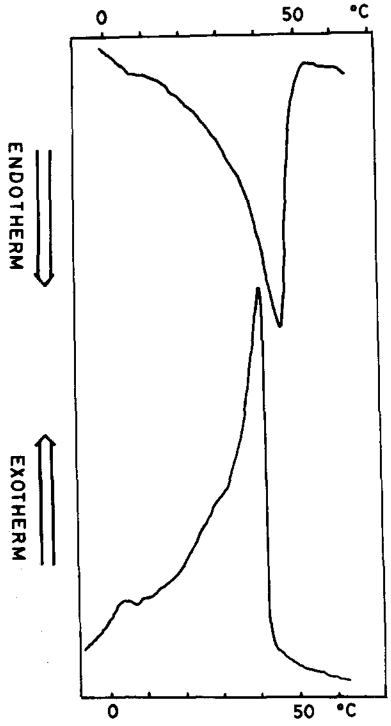

Fig. 3. Differential scanning calorimetric thermograms of PL42 (KCl)/H_2O system (50% w/w). Scanning rate is 10 K · min^{-1}. Upper, heating; lower, cooling.

a distinct difference between the peak temperatures of the two thermograms. A major peak was observed at 46°C (heating) and at 41°C (cooling). The difference became smaller but still existed even at a slower scanning rate (0.3 K · min^{-1}). A minor peak was observed below 10°C. Thus the lipid bilayer exhibits at least two transitions. The difference in the lower phase transition temperature determined by DSC and ^{1}H-NMR may be due to the different conditions of the sample. In addition, there seems to be a gradual change in heat capacity between the two peaks. The shapes of the thermograms observed on both heating and cooling are asymmetric. The asymmetric features resemble the heating thermogram of the elaidate-enriched *E. coli* membrane [15].

Phase transitions of the membrane fraction

Fig. 4 shows the thermograms of the bulk membrane fraction prepared from the same culture of *E. coli* cells as used above. The scanning rate is 0.6 K · min^{-1}. On the first heating, can be seen two large signals, at 43 and 52°C, and a few small signals at approx. 19°C and in the region above 60°C. On the second heating, a large signal at 43°C and a small signal at approx. 19°C were reproducible. When the membrane fraction was retreated with DNAase, the large signal at 52°C was very much reduced in peak height. Therefore, we concluded that the large signal at 52°C is due to the residual pieces of degraded DNA. The

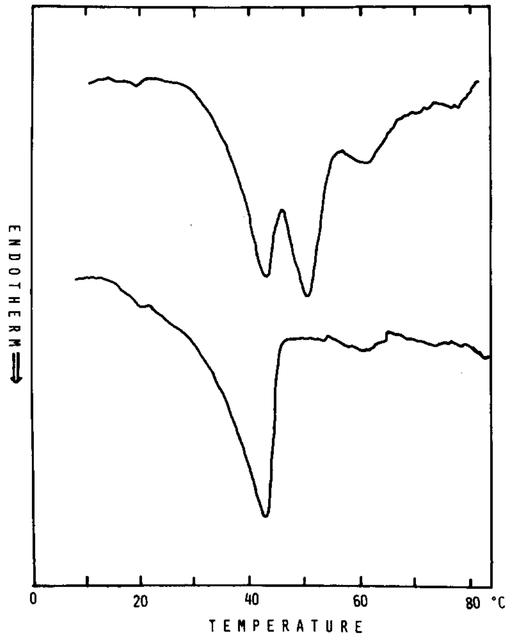

Fig. 4. Differential scanning calorimetric thermograms of the membrane fraction of *E. coli* grown at 42°C under osmotically stabilized conditions (2% KCl). Scanning rate is 0.6 K · min^{-1}. Upper, first run; lower, second run.

small signals in the region above 60° C are presumably due to the membrane proteins, since they undergo irreversible denaturation. The two reproducible peaks at 43 and 19° C are obviously due to the phase transitions of the phospholipid bilayers in the membrane. The profile of the thermogram of the second run shows a close resemblance to that of the extracted phospholipid fraction (PL42 (KCl)). The temperature of the major peak is lower, by approx. 3 K, whereas that of the minor peak is higher, by approx. 10 K, than those observed for the extracted phospholipids. Although the peak temperatures are somewhat modified, it can be concluded that the transition features of the membrane are essentially similar to those of the PL42 (KCl)/H_2O system.

Temperature dependence of β-galactoside transport

It is well known that β-galactoside transport closely correlates with membrane fluidity. The Arrhenius plots of the transport rates have inflection points at the upper and lower boundaries of the phase transition [2,3]. The β-galactoside transport rates in *E. coli* K-12 UFA^{ts} cells which had grown at 42° C in the presence of an osmotic stabilizer (2% KCl) were measured and their Arrhenius plots are shown in Fig. 5. The plots are clearly biphasic and the temperature of the inflection point is 22° C, which is reproducible. The apparent activation energy of β-galactoside transport is almost constant in the region between 5 and 22° C and also in that between 22 and 42° C. The temperature of the inflection point is close to that of the minor phase transition in the membrane fraction.

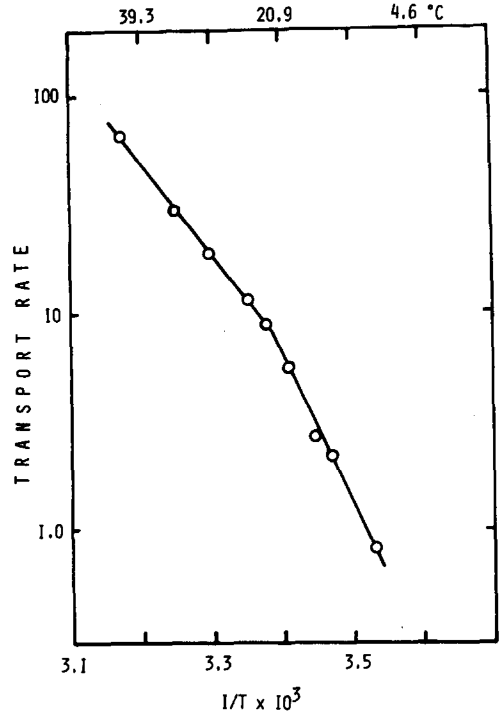

Fig. 5. Arrhenius plot of β-galactoside transport rates in *E. coli* cells grown in osmotically stabilized conditions (2% KCl) at 42°C.

Discussion

The line shape of the ^{1}H-NMR of vesicles is known to depend not only on the local orientational order of the acyl chains but also on the sizes of vesicles [16]. As shown in the electron micrograph of the vesicles taken at room temperature, the sizes range between 200 and 1200 Å [6]. There is no appreciable change in the sizes when photographs were taken at 5°C. Thus the change of the linewidth at approx. 15°C is not mainly due the change in vesicle size. On the other hand, the sizes of vesicles apparently become larger above 40°C. A drastic change of the linewidth at 42°C, however, is not caused by the change in vesicle size, since infrared spectra showed the *trans-gauche* conversion of the acyl chain at approximately that temperature.

Thus the physicochemical methods employed in this work indicate the existence of multiple phase transitions in the phospholipid bilayer prepared from PL42 (KCl). The transition at approx. 42°C is due to the major part of the phospholipids having a very low content of unsaturated fatty acids. The minor transition at a lower temperature is caused by a segregated domain containing a higher amount of unsaturated fatty acids in the gel state. Therefore, the first appearance of the fluid region in the bilayer upon heating is determined by the minor transition. Multiple phase transitions seem a rather general phenomenon in bilayers of *E. coli* phospholipids. A recent publication by

Jackson and Cronan [17] shows DSC thermograms of the bilayers of *E. coli* phospholipids with differing contents of unsaturated fatty acids. The thermogram of the bilayer with 16% unsaturated fatty acids, the lowest one examined, is essentially similar to ours. Although the temperature of the minor peak is slightly higher than ours, this might be due to the difference in the fatty acid composition. With increase of the content of unsaturated fatty acids the intensity of the peak at a lower temperature becomes stronger.

The results of our DSC study on the membrane fraction is consistent with those on the phospholipid extract. The difference in the transition temperature between these two cases may be mainly attributed to the phospholipid-protein interaction in the membrane although the difference in solvent conditions could be partly responsible for it. The membrane fraction contains a small amount of degraded DNA pieces, but they would not affect the phase transition significantly. The membrane fraction used in the present experiment contains both inner and outer membranes. We analysed the phospholipid and fatty acid compositions of both. There is a distinct, but not large, difference between them. Thus we can expect that the major transition takes place in both the inner and outer membranes. Observations of the fatty acid composition of the inner and outer membranes of fatty acid-enriched *E. coli* support the idea [18]. The β-galactoside transport system is localized in the inner membrane [19]. Thus the close correlation of the temprature between the minor phase transition in the membrane fraction and the inflection point in the Arrhenius plots of the β-galactoside transport rates suggests that the minor transition belongs at least to the inner membrane. The multiple phase transitions we hypothesize above probably occur in the inner membrane of *E. coli*.

Our results show that the minor transition can change the apparent activation energy of β-galactoside transport. This fact suggests that the key proteins of the transport system are localized in or around the domain with a high content of unsaturated fatty acids in the gel sate. In fact, Letellier et al. and Heerikhuizen et al. observed localization of membrane proteins [4,20]. If a certain amount of the fluid region of the lipid bilayer is vital for membrane functions, the temperature of the minor phase transition would be relevant to the lowest temperature for cell growth. We examined this by observing the growth rate of cultures shifted from 42°C to lower temperatures. At the shift to 35 and 30°C, growth became progressively slower. At 25°C, growth resumed after a lag of approx. 1 h, indicating that unsaturated fatty acid synthesis is restored. The evidence shows that the cell can still grow without any lag time after the sudden decrease of the temperature by 12°C, although the growth rate is lower. Since the growth temperature is still above the minor phase transition temperature, the fluid region in the membrane seems to be necessary for growth, which agrees with the conclusion of Jackson and Cronan [17]. The cell enriched with elaidic acid, showing no minor transition [15], could not grow below 37°C [21]. This fact suggests that the minor transition at the low temperature enlarges the temperature range over which a rapid adaptation to the change of the growth temperature can occur. As is well known, slow adaptation occurs by regulation of the pathway of fatty acid synthesis [22,23].

Acknowledgements

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